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Enantioselective separation of racemates using CHIRALPAK IG amylose-based chiral stationary phase under normal standard, non-standard and reversed phase high performance liquid chromatography

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ABSTRACT

We have previously reported on the solvent versatility of immobilized amylose and cellulose-based chiral stationary phases in enantioselective liquid chromatographic separation of racemates. The studies were mainly focusing on the *tris* substituted 3,5-dimethylphenylcarbamate polysaccharide-based chiral stationary phases namely CHIRALPAK IA® [Amylose *tris* (3,5-dimethylphenylcarbamate)] or ADMPC and CHIRALPAK IB® [Cellulose *tris* (3,5-dimethylphenylcarbamate)] or CDMPC. Here we focus on the application of the recently introduced amylose *tris* (3-chloro-5-methylphenylcarbamate) or ACMPC and brand name CHIRALPAK IG® with a chlorine substituent replacing the methyl group in CHIRALPAK IA®. This was investigated for the enantioslective separation of different classes of pharmaceuticals namely β- and α-blockers, anti-inflammatory and antifungal drugs, norepinephrine-dopamine reuptake inhibitor, catecholamines, sedative hypnotics, anti-histaminics, anticancer drugs, antiarrhythmic drugs, flavonoids, amino acids, alpha-2 adrenergic agonist, adrenaline and miscellaneous.A brief comparison between CHIRALPAK IG® and CHIRALPAK IA® under normal standard, non-standard and reversed mobile phase is demonstrated. The results revealed the versatility of the CHIRALPAK IG® column, its compatibility with a wide ranges of solvent and operation modes and its ability to separate chiral compounds not separated with other amylose based chiral stationary phases.

1. Introduction

Many pharmaceutics and herbicides are chiral. They exist as two incongruent stereoisomers called enantiomers. As optical isomers, they rotate linearly polarized light in opposite directions although they are generally known to have similar physical properties (eg, melting point, hydrophobicity, etc) and they can behave quite differently to one another in a chiral (asymmetric) environment. Since biological processes tend to involve chiral chemicals (eg. enzymes), chirality constitutes an important topic in drug development [1]. The United States Food and Drug Administration (FDA) requires toxicology testing for racemates only, regardless of industry plans to market a single isomer. In case of unexpected or significant toxicity is found in the racemate, FDA suggests querying the agency on whether similar studies are required for individual enantiomers. In such case, the FDA requires that only the active drug enantiomer (*the eutomer*) is produced by an enantioselective access (*e.g.*, via asymmetric synthesis, resolution via diastereomers, kinetic resolution, enzyme catalysis or chirality pool approach). The inactive enantiomer (*the distomer*) constitutes 'isomeric ballast' or it may be highly toxic. In the case of thalidomide, one enantiomer possessed the required therapeutic effect, while the other was eventually shown to be teratogenic causing birth defects in the unborn babies. While the use of enantiomerically pure drugs may appear to be a viable solution to such a problem, configurationally unstable stereoisomers like thalidomide may interconvert (known variously as enantiomerization, enantiomeric inversion or racemisation) [2]. The thalidomide tragedy was entirely avoidable, had the physiological properties of the individual thalidomide forms been identified, separated and tested prior to commercialization.

Enantioselective chromatography has been well documented as a powerful, contemporary and practical technique for the chiral separation of racemic drugs, food additives, agrochemicals, fragrances and chiral pollutants [1,2]. This technique is several steps ahead of other previously reported methods to access pure

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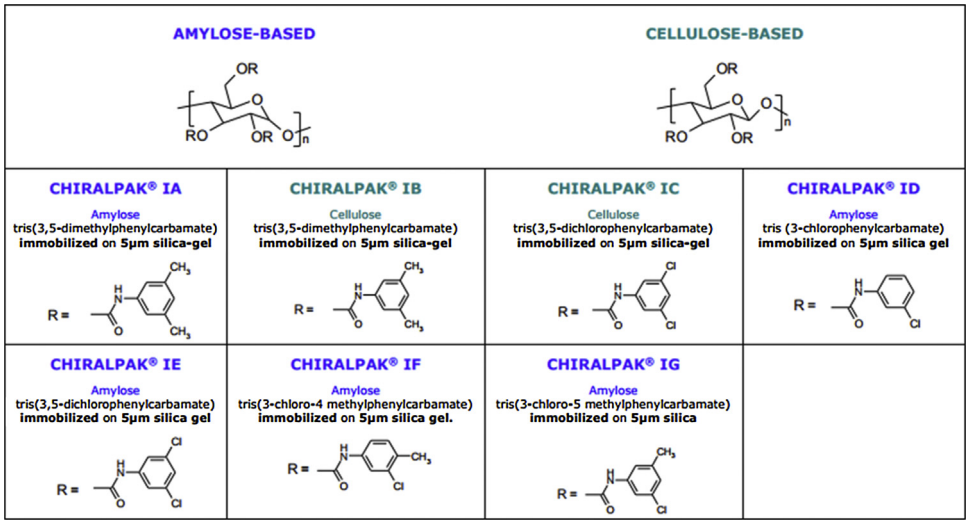

Fig. 1. Chemical structures of CHIRALPAK® amylose and cellulose based chiral stationary phases.

enantiomers; including synthesis from a chirality pool, asymmetric synthesis from pro-chiral substrates and the resolution of racemic mixtures [3]. The separation of racemic mixtures has been considered as the most feasible method for industrial applications compared to the time consuming and expensive synthetic approaches [4]. Remarkable developments have occurred in enantioselective chromatography since the first chiral separation of enantiomers using optically active stationary phase in the mid-sixties [5]. Following this development, several subclasses have emerged as well established chromatographic techniques with outstanding applications in chiral separation like electrochromatography (EC), supercritical fluid chromatography (SFC), counter current chromatography (CCC), gas chromatography (GC), and high performance liquid chromatography (HPLC) [6]. The chiral selectors used as stationary phases in liquid chromatography play a crucial role in the separation efficiency and the column backpressure governing the entire separation [1].

Most enantioselective separations are performed by direct resolution using a chiral stationary phase (CSP) where the chiral selector is adsorbed, attached, bound, encapsulated or immobilized to an appropriate support to make a CSP. The enantiomers are resolved by the formation of temporary diastereomeric complexes between the CSP and the analyte. Yet, thousands of CSPs have been reported, with more than one hundred commercialized [7]. Among the existing CSPs, those prepared from polysaccharides such as cellulose and amylose, attract more attention due to their powerful separation capability [8–18]. In general, the developments of chemically post-modified polysaccharides are the mainstream trend in the commercial and non-commercial chiral stationary phases. Out of the commercially available polysaccharide-based chiral stationary phases, cellulose and amylose were adsorbed, bonded, encapsulated or immobilized [19–26]. Of the amylose derivatives, the coated *tris* (3,5-dimethylphenylcarbamate) known as CHIRALPAK AD® has been widely and effectively used in chiral separation. However, it is not compatible to all eluents solvents, in particular, non-standard organic solvents such as ethyl acetate (EtOAc), tetrahydrofuran (THF), methyl *tert*-butyl ether (MtBE), dichloromethane (DCM) and chloroform, in which the polysaccharide derivatives can be dissolved or swollen. To widen the selection of solvents, the polysaccharide derivatives have been immobilized/bonded onto a silica matrix and have been extensively used as chiral stationary phases in non-standard organic solvents. Such immobilization of the polymeric chiral selector is considered as an efficient approach to confer a universal solvent versatility [27–32]. Several immobilized phases have been commercialized (Fig. 1). For examples CHIRALPAK IA®: Amylose *tris* (3,5-dimethylphenylcarbamate); CHIRALPAK IB®: Cellulose *tris* (3,5-dimethylphenylcarbamate); CHIRALPAK IC®: Cellulose *tris* (3,5-dichlorophenylcarbamate); CHIRALPAK ID®: Amylose *tris* (3-chlorophenylcarbamate); CHIRALPAK IE®: Amylose *tris* (3,5-dichlorophenylcarbamate) and CHIRALPAK IF®: Amylose *tris* (3-chloro-4-methylphenylcarbamate) have been extensively studied and proved to be solvents versatile in the enantiomeric separation of racemates [3,4]. Most recently CHIRALPAK IG®: Amylose *tris* (3-chloro-5- methylphenylcarbamate) with a chlorine substituent replacing the methyl group in CHIRALPAK IA® was introduced. Here we focus on the solvents versatility of CHIRALPAK IG® and the enantioselective separations of racemates (Fig. 2) under non-standard organic solvents and reversed phase chromatographic conditions. A brief comparison with CHIRALPAK IA® showing the effect of chlorine substituent in CHIRALPAK IG® on the enantiomeric separation of racemates is also demonstrated.

2. Materials and methods

2.1. Instrumentation

Conventional HPLC analysis was carried out using a Prominence Shimadzu System that consists of an LC-20 AD VP pump (Kyoto, Japan), SIL- 20AHT auto sampler, a GL Science UV–vis detector model MU 701 UVVIS (Tokyo, Japan), and a Shimadzu CDM-20A communications bus module (Kyoto, Japan). All analyses were performed at room temperature. CHIRALPAK IG® (4.6 mm ID × 250 mm, 5 µm silica gel) was supplied by Daicel (Tokyo, Japan).

2.2. Chemicals and reagents

All solvents were HPLC grade purchased from Sigma-Aldrich (St. Louis, MO, USA). Most of the tested compounds (Fig. 2) were also purchased from Sigma-Aldrich (St. Louis, MO, USA) namely Propranolol **1**, Naproxen **3**, Flurbiprofen **4**, Indoprofen **5**, Miconazole **8**, Nomifensine **10**, Arterenol **11**, Normetanephrine **12**, Ifosfamide **15**, Tocainide **16** Propafenone **17**, Glutamic acid monohydrate **20**, Tyrosin **21**, Phenylalanine **22**, α-Methyl DOPA **23**, Epinephrine **24**, 1-Acenaphthenol **25** and 4-Hydroxy-3-methoxymandelic acid **26**. On the hand, Naftopidil **2** was purchased from Boehringer Mannheim (Mannheim, Germany), Cizolirtine **6** was purchased from American Custom Chemicals Corp., (San Diego, CA, USA),

Carprofen **7** and Sulconazole **9** were purchased from AK Scientific (Union, CA, USA), Aminoglutethimide **13** was purchased from CIBA GEIGY (Basel, Switzerland), Chlorpheneramine **14** was purchased from Research Biochemicals International (Natick, MA, USA), Flavanone **18 and** 6-Hydroxyflavanone **19** were purchased from Alfa Aesar (Ward Hill, MA, USA). 1-Indanol **27** was purchased from Fluka Chemical (Milwaukee, WI, USA). 1-Phenyl-2,2,2-trifluorethanol **28** was purchased from Sigma-Aldrich Switzerland.

Classification of the investigated racemates and their purities are as listed below:

Classification	Durg	Purity & Supplier
β-blocker	Propranolol **1**	99%, Sigma-Aldrich, USA
α-Blockers	Naftopidil **2**	NA, Sigma-Aldrich, USA
Anti-inflammatory drugs	Naproxen **3**	NA, Sigma-Aldrich, USA
	Flurbiprofen **4**	NA, Sigma-Aldrich, USA
	Indoprofen **5**	NA, Sigma-Aldrich, USA
	Cizolirtine **6**	NA, American Custom Chemicals Corp., USA
	Carprofen **7**	98%, AK Scientific, USA
Antifungal drugs	Miconazole **8**	98% Sigma-Aldrich, USA
	Sulconazole **9**	NA, AK Scientific, USA
Norepinephrine-dopamine reuptake inhibitor	Nomifensine **10**	NA, Sigma-Aldrich, USA
Catecholamines	Arterenol **11**	97%, Sigma-Aldrich, USA
	Normetanephrine**12**	98%, Sigma-Aldrich, USA
Sedative hypnotic	Aminoglutethimide**13**	NA, CIBA GEIGY, Switzerland
Anti-histaminic	Chlorpheneramine**14**	NA, Research Biochemicals International, USA
Anticancer drug	Ifosfamide **15**	98%, Sigma-Aldrich, USA
Antiarrhythmic drugs	Tocainide **16**	98%, Sigma-Aldrich, USA
	Propafenone **17**	NA, Sigma-Aldrich, USA
Flavonoids	Flavanone **18**	98%, Alfa Aesar, USA
	6-Hydroxyflavanone**19**	98%, Alfa Aesar, USA
Amino acids	Glutamic acid monohydrate **20**	98%, Sigma-Aldrich, USA
	Tyrosin **21**	99%, Sigma-Aldrich, USA
	Phenylalanine **22**	99%, Sigma-Aldrich, USA
Alpha-2 adrenergic agonist	a-Methyl DOPA **23**	NA, Sigma-Aldrich, USA
Adrenaline	Epinephrine **24**	NA, Sigma-Aldrich, USA
Miscellaneous	1-Acenaphthenol **25**	99%, Sigma-Aldrich, USA
	4-Hydroxy-3-methoxymandelic acid **26**	98%, Sigma-Aldrich, USA
	1-Indanol **27**	98%, Fluka Chemika, USA
	1-Phenyl-2,2,2-trifluorethanol **28**	98%, Sigma-Aldrich, Switzerland

2.3. Sample preparations

Stock solutions of the racemic analytes at concentrations of 1 mg/mL in filtered HPLC-grade 2-propanol were prepared, filtered through Sartorius Minisart RC 15 0.2-μm pore size filters (Goettingen, Germany) and further used for analysis without dilution; the injection volume was 1 μL.

2.4. HPLC conditions

The enantioselective analyses were conducted using standard normal mobile phase comprised of *n*-hexane in combination with 2-propanol (2-PrOH) or ethanol (EtOH) and non-standard normal phase namely tetrahydrofuran (THF), dichloromethane (DCM) and methyl *tert*-butyl ether (MtBE). Reversed mobile phase consisted of acetonitrile (ACN) and water (H_2O) mixture. The additives TEA and TFA were added in both normal and reversed mobile phases. UV analyses were performed at fixed wavelength (254 nm) for all compounds.

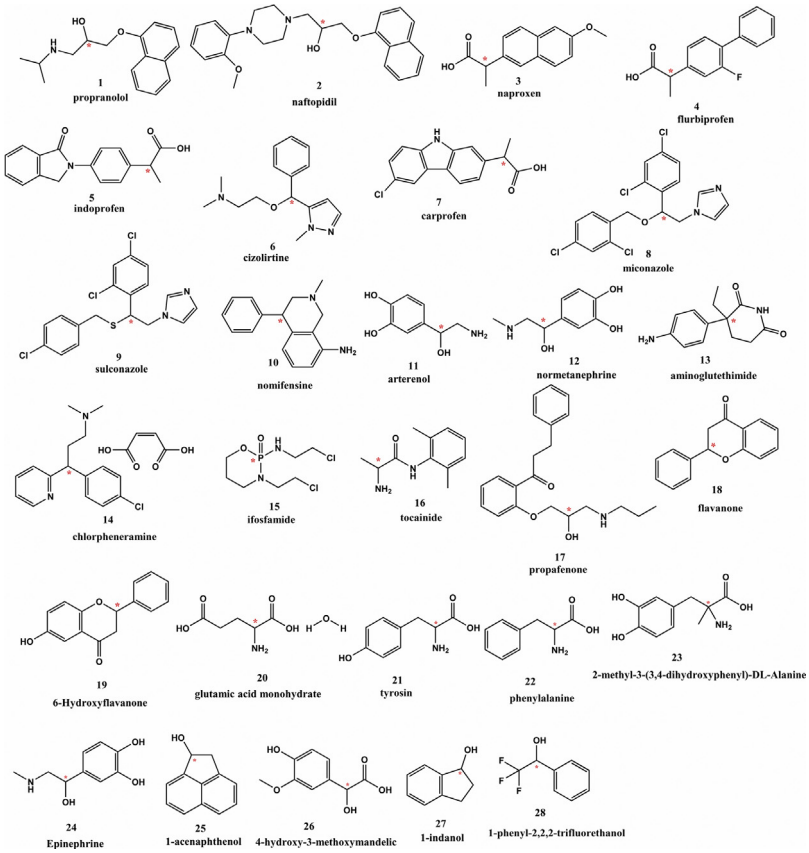

Fig. 2. Chemical structures of a set of racemates investigated for their enantioselective separation on CHIRALPAK IG®.

3. Results and discussion

The well-known coated amylose *tris* (3,5-dimethylphenylcarbamate) ADMPC (CHIRALPAK AD®) in which the amylose derivative is physically coated on 5 or 10 μm silica particles has been widely and effectively used in chiral separation of racemates in high performance liquid chromatography. Its immobilized version namely CHIRALPAK IA® introduced ten years ago showed excellent solvent versatility and enantioselectivity in normal standard and non-standard organic mobile phases [27–32]. More recently, this phase showed promising enantioselectivity under HILIC and reversed phase modes as well [33]. In CHIRALPAK IA®, the chiral selector is immobilized/bonded onto 5 μm silica particles. The replacement of one donating methyl group with a withdrawing chlorine substituent of the ADMPC has resulted in the commercialization of a new immobilized phase namely amylose *tris* (3-chloro-5-methylphenylcarbamate) known as CHIRALPAK IG® or ACMPC. Here we demonstrate the solvent versatility and enantioselectivity of the new phase CHIRALPAK IG® under normal standard and non-standard organic phase as well as reversed phase chromatographic conditions. A brief comparison with CHIRALPAK IA® showing the effect of the donating methyl *vs* withdrawing chlorine substituent in amylose derivatives on the enantioselectivity is briefly demonstrated.

3.1. Chiral separation under normal standard and non-standard organic mobile phase

The initial mobile phase selected for the enantioselective separation of racemates 1–28 (Fig. 1) was a binary mixture of standard organic solvents consisting of *n*-hexane/ethanol screened from 90:10 to 10:90 *v*/*v* at 1 ml/min flow rate on CHIRALPAK IG® at fixed UV detection 245 nm. Out of the twenty eight compounds screened, fifteen compounds namely **1**, **3–8**, **10, 12, 14, 16–19 and 25–28** were baseline separated under either 90:10 or 80:20 v/v *n*-hexane/ethanol, respectively (Table 1 and Fig. 3). No baseline separation was achieved for **2**, **9**, **11**, **13**, **15** and **20–24**. Replacing ethanol (EtOH) with 2-propanol (2-PrOH) resulted in the baseline separation of **4**, **6**, **7**, **8**, **10**, **12**, **16–19**, **25 and 27** under either 90:10, 80:20, 70:30 or 60:40 v/v *n*-hexane/2-PrOH (Table 1). Comparing 2-PrOH with ethanol in mobile phase composition and in terms of enantioselective separation, resolution Rs and separation factor α, ethanol in mobile phase composition was superior than 2-PrOH. Thus, **1**, **3**, **5**, **14**, **26** and **28** were all separated under *n*-hexane/ethanol which wasn't the case in *n*-hexane/2-PrOH implying that ethanol works better with the 3-chloro substituted amylose in amylose *tris* (3-chloro-5-methylphenylcarbamate) or CHIRALPAK IG®. It is noteworthy that the retention is generally shorter with ethanol than 2-PrOH or when using higher alcohol contents in relation to *n*-hexane in mobile phase composition (Table 2 and Fig. 3). To widen the choice of solvents in an attempt to enhance the separation or resolve the unresolved compounds under standard solvents above; dichloromethane (DCM), tetrahydrofuran (THF) or methyl *tert*-butyl ether (MtBE) were used before combination with standard organic solvent. The addition of non-standard solvents in mobile phase composition enhanced the resolution R_s and separation factor α of several tested racemates (Table 1). For example, in case of **6**, the resolution R_s jumped from R_s 1.47 and separation factor α 1.38 in standard solvents namely *n*-hexane/2-PrOH 90:10 v/v, respectively and R_s 3.83 and α 1.19 in *n*-hexane/EtOH 90:10 *v*/*v* to R_s 5.13 and separation factor α 3.35 when using non-standard solvent in excess in mobile phase composition (MtBE 98% v) in combination with ethanol (EtOH 2% v) or MtBE/EtOH 98:2% v/v. Of particular interest, compound **11** which wasn't resolved under any standard solvents 'combination investigated in this study was baseline separated under excess of

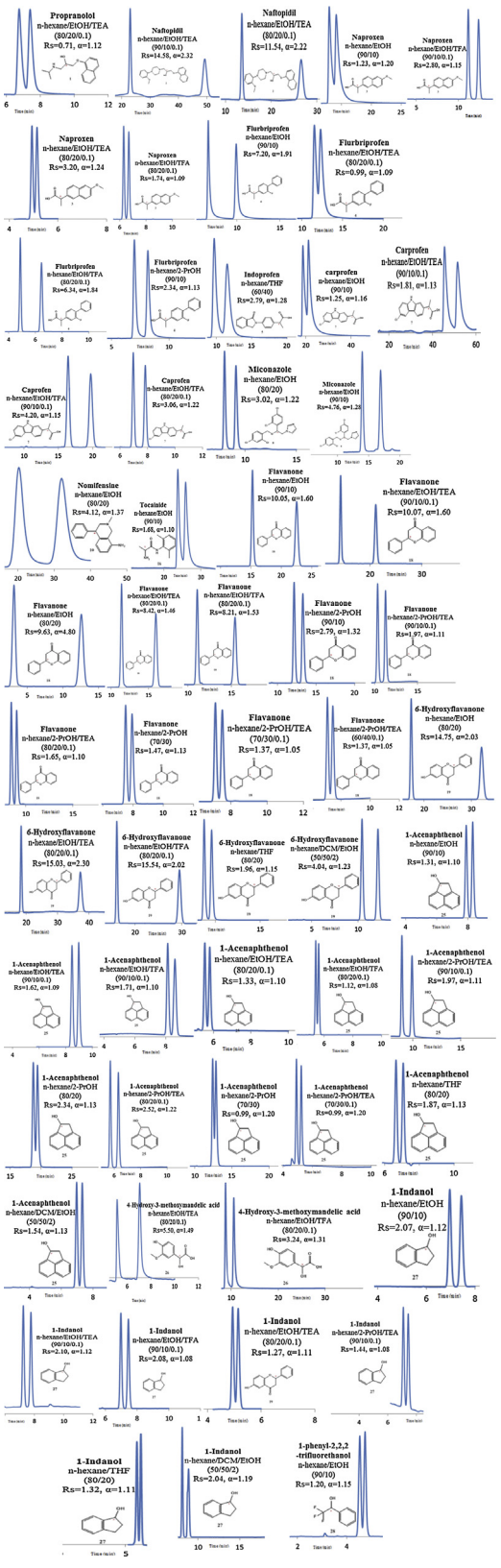

Fig. 3. UV traces/Chromatograms for the enantioselective separation of racemates under normal standard and non-standard mobile phase.

Table 1
The resolution Rs and separation factor α for the enantioselective separation of racemates under normal standard and non-standard mobile phase condition (Rs <1 = not separated, Rs > 1 = separated.

IG			Normal solvents						Additives		IA	
	Standard solvents		Nonstandard solvents									
	Rs	α	n-Hexane	2-PrOH	EtOH	THF	DCM	MtBE	TEA	TFA	Rs	α
1	0.621	1.137	80		20						NS	NS
	1.202	1.152	90		10						NS	NS
	0.711	1.12	80		20				0.1		NS	NS
	0.278	1.12	80		20					0.1	NS	NS
2	NS	NS	90		10						0.31	1.18
3	1.243	1.122	80		20						NS	NS
	1.232	1.119	90		10						NS	NS
	3.196	1.236	80		20				0.1		NS	NS
	5.411	1.384	90		10				0.1		NS	NS
	1.745	1.091	80		20					0.1	NS	NS
	2.799	1.151	90		10					0.1	NS	NS
	0.678	1.149	80			20					NS	NS
	2.035	1.634			2			98			NS	NS
4	2.341	1.129	90	10							NS	NS
	1.469	1.134	70	30							NS	NS
	2.434	1.125	90	10					0.1		NS	NS
	1.652	1.104	80	20					0.1		NS	NS
	1.367	1.054	60	40					0.1		NS	NS
	9.632	4.803	80		20						NS	NS
	10.05	1.597	90		10						NS	NS
	8.423	1.462	80		20				0.1		NS	NS
	8.214	1.533	80		20					0.1	NS	NS
	9.202	1.931	90		10					0.1	NS	NS
	0.798	1.132	80			20					NS	NS
	1.15	1.338	50		0.2		50				NS	NS
5	3.361	1.497	80		20					0.1	NS	NS
	2.793	1.275	60			40					NS	NS
	1.413	1.347			2			98			NS	NS
6	1.47	1.38	90	10							NS	NS
	2.361	1.134	80		20				0.1		NS	NS
	3.835	1.19	90		10				0.1		NS	NS
	5.131	3.354			2			98			NS	NS
	2.391	1.684			60			40			NS	NS
7	0.897	1.141	90	10							NS	NS
	1.454	1.15	80	20							NS	NS
	0.399	1.121	80	20					0.1		NS	NS
	0.647	1.347	60	40					0.1		NS	NS
	1.435	1.173	80		20						NS	NS
	1.247	1.16	90		10						NS	NS
	0.778	1.067	80		20				0.1		NS	NS
	1.813	1.131	90		10				0.1		NS	NS
	3.058	1.218	80		20					0.1	NS	NS
	4.197	1.247	90		10					0.1	NS	NS
	1.289	1.198	50		0.2		50				NS	NS
	1.123	1.095	70	30							NS	NS
8	1.141	1.053	80	20					0.1		NS	NS
	3.018	1.224	80		20						NS	NS
	4.758	1.282	90		10						NS	NS
	3.397	1.292	80		20				0.1		NS	NS
	5.268	1.293	90		10				0.1		NS	NS
9	2.503	1.242	80	20					0.1		NS	NS
	1.964	1.281	60	40					0.1		NS	NS
10	4.116	1.372	80	20							NS	NS
	3.74	1.672	70	30							NS	NS
	4.65	1.454	80	20					0.1		NS	NS
	2.528	1.399	60	40					0.1		NS	NS
	2.615	1.772	80		20						NS	NS
11	1.248	1.28			60			40			NS	NS
12	1.14	1.104	80	20					0.1		NS	NS
	0.671	1.604	80		20				0.1		NS	NS
	0.891	1.041	90		10				0.1		NS	NS
13	5.136	2.121			2			98			6.94	3.429
14	3.327	1.286	80		20				0.1		NS	NS
16	0.415	1.123	80	20							NS	NS
	0.648	1.104	70	30							NS	NS
	0.737	1.132	80	20					0.1		NS	NS
	5.02	1.152	60	40					0.1		NS	NS
	1.677	1.101	90		10						NS	NS
	1.338	1.083	80		20				0.1		NS	NS
	2.369	1.119	90		10				0.1		NS	NS
	0.944	1.188	80		20					0.1	NS	NS
	1.084	1.149	80			20					NS	NS

Table 1 (*Continued*)

IG	Standard solvents		Normal solvents						Additives		IA	
				Nonstandard solvents								
	Rs	α	n-Hexane	2-PrOH	EtOH	THF	DCM	MtBE	TEA	TFA	Rs	α
	2.392	1.518			60			40			NS	NS
17	0.711	1.041	90	10							NS	NS
	4.556	1.699	80	20							NS	NS
	5.138	1.571	70	30							NS	NS
	8.887	2.012	80	20					0.1		NS	NS
	7.984	2.194	60	40					0.1		NS	NS
	9.048	1.636	90		10						NS	NS
	10.642	1.881	80		20				0.1		NS	NS
	0.879	1.15	90		10					0.1	NS	NS
	1.809	1.126	70			30					NS	NS
18	2.787	1.321	90	10							1.645	1.114
	1.972	1.108	90	10					0.1		NS	NS
	7.2	1.907	90		10						15.614	2.32
	8.423	1.462	80		20				0.1		NS	NS
	10.05	1.597	90		10				0.1		NS	NS
	8.214	1.533	80		20					0.1	NS	NS
	9.967	1.595	90		10					0.1	NS	NS
	4.724	1.673			2			98			1.51	1.468
	2.354	1.652			60			40			NS	NS
19	1.453	1.115	90	10							0.599	1.17
	2.656	1.159	80	20							NS	NS
	1.191	1.142	70	30							NS	NS
	1.323	1.126	90	10					0.1		NS	NS
	1.329	1.16	80	20					0.1		NS	NS
	0.985	1.146	70	30					0.1		NS	NS
	14.749	2.029	80		20						NS	NS
	15.026	2.304	80		20				0.1		NS	NS
	15.536	2.022	80		20					0.1	NS	NS
	1.919	1.141	70			30					NS	NS
	1.958	1.147	80			20					NS	NS
	4.038	1.231	50		0.2		50				NS	NS
	7.971	3.784			2			98			0.947	1.32
	3.098	2.544			60			40			NS	NS
25	1.039	1.031	90	10							1.516	1.092
	1.14	1.203	80	20							NS	NS
	0.99	1.202	70	30							NS	NS
	3.244	1.18	90	10					0.1		NS	NS
	2.519	1.217	80	20					0.1		NS	NS
	1.708	1.274	70	30					0.1		NS	NS
	1.334	1.18	60	40					0.1		NS	NS
	1.306	1.101	80		20						NS	NS
	1.68	1.095	90		10						1.041	1.071
	1.328	1.103	80		20				0.1		NS	NS
	1.619	1.091	90		10				0.1		NS	NS
	1.116	1.082	80		20					0.1	NS	NS
	1.711	1.098	90		10					0.1	NS	NS
	0.661	1.066	60			40					NS	NS
	1.87	1.128	80			20					NS	NS
	1.538	1.126	50		0.2		50				NS	NS
	1.324	1.332			2			98			NS	NS
26	5.505	1.493	80		20				0.1		NS	NS
	3.242	1.309	80		20					0.1	NS	NS
	5.075	1.665	90		10					0.1	NS	NS
27	1.195	1.035	90	10							1.896	1.137
	1.107	1.291	80	20							NS	NS
	1	1.29	70	30							NS	NS
	1.441	1.085	90	10					0.1		NS	NS
	0.971	1.065	80	20					0.1		NS	NS
	0.886	1.029	70	30					0.1		NS	NS
	0.722	1.037	60	40					0.1		NS	NS
	1.371	1.119	80		20							
	2.068	1.123	90		10						1.658	1.133
	1.271	1.114	80		20				0.1		NS	NS
	2.098	1.125	90		10				0.1		NS	NS
	1.133	1.095	80		20					0.1	NS	NS
	2.081	1.078	90		10					0.1	NS	NS
	1.324	1.113	80			20					NS	NS
	2.039	1.193	50		0.2		50				NS	NS
	NS	NS			2			98			0.687	1.175
28	NS	NS	90	10							0.906	1.106
	0.621	1.137	80		20						NS	NS
	1.202	1.152	90		10						NS	NS
	0.711	1.12	80		20				0.1		NS	NS
	0.278	1.12	80		20					0.1	NS	NS

Table 2
The resolution Rs and separation factor α for the enantioselective separation of racemates under reversed mobile phase condition.

Reversedmobilephase					
	IG			IA	
	Rs	α	ACN/H2O/0.1TEA	Rs	α
2	NS	NS	60/40	1.429	1.097
4	16.801	2.637	60/40	NS	NS
	12.384	2.719	80/20	NS	NS
8	5.831	1.461	60/40	NS	NS
12	1.325	1.179	60/40	NS	NS
13	NS	NS	60/40	2.275	1.285
14	0.965	1.084	60/40	NS	NS
	1.1	1.071	80/20	NS	NS
16	1.443	1.09	60/40	NS	NS
	1.496	1.109	80/20	NS	NS
17	4.549	1.399	60/40	NS	NS
	4.216	1.385	80/20	NS	NS
18	NS	NS	60/40	10.91	1.678
19	11.174	2.965	40/60	NS	NS
	7.721	3.214	60/40	5.563	2.822
	5.825	2.441	80/20	NS	NS
25	1.125	1.055	40/60	NS	NS
26	1.117	1.247	60/40	NS	NS
27	1.897	1.133	40/60	NS	NS
	0.906	1.07	60/40	NS	NS

non-standard organic solvent (MtBE 98% v) in combination with ethanol (EtOH 2% v) or MtBE/EtOH 98:2 *v*/*v*, respectively with resolution R_s 5.13 and separation factor α 2.12. Similarly compound **13** was only separated under non-standard organic mobile phase composition consisting of MtBE/EtOH 98:2 *v*/*v*. Better separations were achieved in non-standard solvents when compared to similar separation under standard organic solvents for compounds **3**, **6** and **16** (Table 1 and Fig. 3). One can conclude that polarity plays a role in the chiral recognition of CHIRALPAK IG®. For example, ethanol with polarity index 5.2 works well in combination with *n*-hexane or MtBE while 2-PrOH with polarity index 3.9 is less sensible in terms of enantioseparation under standard and non-standard organic solvents. Another factor might be the amendment of the stereo environment of the chiral cavities in amylose derivatives is favourable in presence of ethanol for the enantioseparation of the investigated racemates.

3.2. Chiral separation under reversed phase

Although the use of reversed phase in amylose and cellulose-based as CSPs in enantioselective liquid chromatography is limited, there are few recently reported studies [22,34–39]. The choice of reversed phase was based on its economic and environmental benefits. Thus, the enantioselective separation was investigated using reversed phases including acetonitrile (ACN) and water (H_2O) mixture ranging from 10–90% (*v*/*v*) (Table 2 and Fig. 4). Few baseline separations were achieved under acetonitrile condition for compounds **4**, **8**, **12**, **14**, **16**, **17**, **19**, **25**, **26** and **27**. Of particular interest compound **4** was baseline separated with unpreceded resolution of Rs 16.80 and separation factor α 2.63 under ACN/H_2O 60:40 *v*/*v* in presence of 0.1% TEA in mobile phase composition. Similarly, in case of **8**; Rs 5.83 and separation factor α 1.46 were superior to other separations achieved under standard and non-standard organic solvents (Table 2 and Fig. 4). Compound **12** which was moderately separated under standard and non-standard solvents, was baseline separated under reversed phase condition (ACN/H_2O/TEA 60:40:0.1% v/v) with superior Rs 1.35 and α 1.46.

3.3. Methyl vs chlorine substituent in CHIRALPAK IA® vs CHIRALPAK IG®

In an attempt to study the effect of the introduction of the withdrawing chlorine group instead of donating methyl group in the third position of amylose *tris* (3,5-dimethylphenylcarbamate) or ADMPC known as CHIRALAPAK IA® to make the amylose *tris* (3-chloro-5-methylphenylcarbamate) known as CHIRALPAK IG®, a brief comparison between CHIRALPAK IA® vs CHIRALPAK IG® took place under standard, non-standard and reversed phase mobile phase composition for the enantioselective separation of selected racemates (Fig. 2). As previously demonstrated above, in terms of the resolution R_s and separation factor α, *n*-hexane/EtOH 90:10 *v*/*v* mixture was the best performing mixture of standard solvents in mobile phase composition. This mobile phase mixture was chosen in the comparison study for the enantioselective resolution of racemates under CHIRALPAK IA®. Comparing with CHIRALPAK IG®, under similar condition, only compounds **2** (R_s 0.31, α 1.11), **18** (R_s 15.61, α 2.32), **25** (R_s 1.04, α 1.07) and **27** (R_s 1.65, α 1.13) were partially or base-line separated under *n*-hexane/EtOH 90:10 *v*/*v* mobile phase. Moving to *n*-hexane/2-PrOH 90:10 *v*/*v* instead of *n*-hexane/EtOH 90:10 *v*/*v* in mobile phase composition resulted in the partial or base-line separation of **18** (R_s 1.64, α 1.11), **19** (R_s 0.59, α 1.17), **25** (R_s 1.51, α 1.09), **27** (R_s 1.89, α 1.13) and **28** (R_s 0.90, α 1.10). In terms of resolution Rs and separation factor α, CHIRALPAK IG® was superior than CHIRALPAK IA® when operating under *n*-hexane/EtOH 90:10 *v*/*v* or *n*-hexane/2-PrOH 90:10 *v*/*v* in mobile phase composition. When using non-standard solvent in excess in mobile phase composition *e.g.* *n*-hexane/MtBE 2/98% *v*/*v*, only two compounds were separated on CHIRALPAK IA® namely compounds **18** (R_s 1.51, α 1.46) and **27** (R_s 0.68, α 1.17). Moving to MtBE/EtOH 98/2% v/v, only four compounds were separated under CHIRALPAK IA® namely **13**, **18**, **19** and **26** comparing to seven compounds separated under CHIRALPAK IG® (**3**, **5**, **6**, **13**, **18**, **19**, **25** and **26**). It is noteworthy that the resolution Rs and separation factor α were all better on CHIRALPAK IG® (Table 1). The results align with previous finding about the chiral recognition of the regioselective substituted polysaccharide derivatives [8]. The different chiral recognition abilities may be ascribed to the electronic effect of substituents namely the withdrawing chlorine group versus the donating methyl group which in turn can alter the polarity and the 3D structure of the polymer.

Under reversed mobile phase composition namely ACN/H_2O/TEA 60:40:0.1 *v*/*v*/*v* respectively, only compounds **2** (R_s 1.42, α 1.09), **13** (R_s 2.27, α 1.28), **18** (R_s 10.91, α 1.67), and **19** (R_s 5.56, α 2.82) were baseline separated on CHIRALPAK IA® comparing to compounds **4, 8**, **12**, **14**, **16**, **17**, **19**, **25**, **26**, and **27**

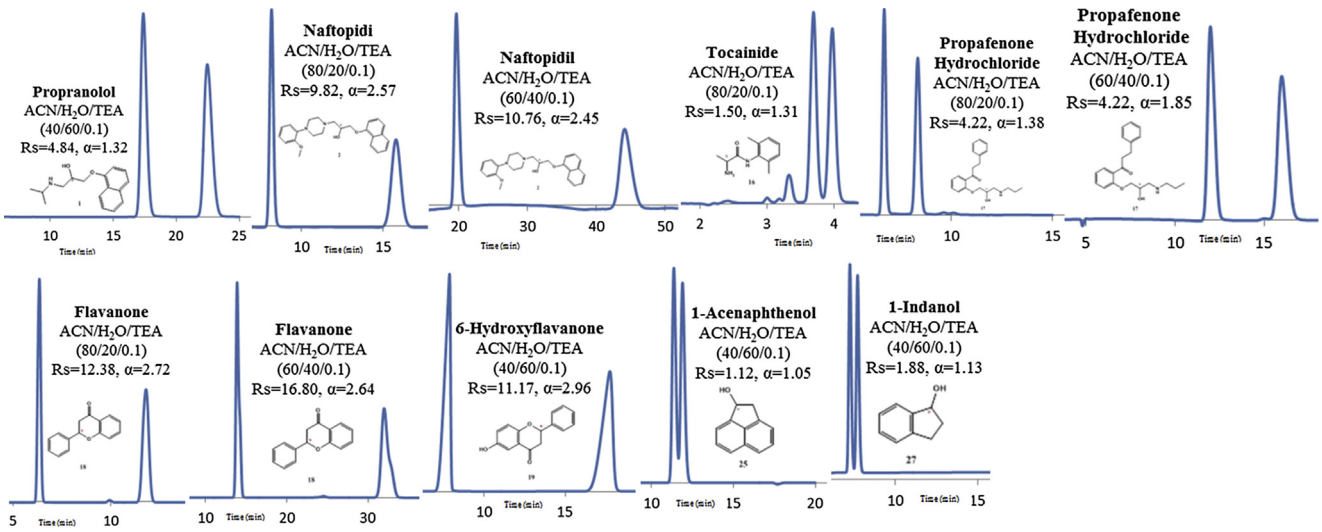

Fig. 4. UV traces/Chromatograms for the enantioselective separation of racemates under reversed mobile phase (ACN/H_2O).

separated on CHIRALPAK IG®. It is noteworthy to mention that compounds **2**, **13**, **18** were not previously separated on CHIRALPAK IG® under similar conditions (Table 2).

The enantioselective separation under non-standard solvents' mobile phase revealed that the combination of *n*-hexane with MtBE works best where ten compounds (**3**, **5**, **6**, **11**, **13**, **16**, **18**, **19**, **25** and **27**) were separated comparing with *n*-hexane/THF with eight compounds separated (**3**, **4**, **5**, **16**, **17**, **19**, **25** and **27**) and *n*-hexane/DCM with only four compunds separated (**4**, **7**, **25** and **27**).

4. Conclusions

The solvents versatility of CHIRALPAK IG® has been demonstrated. The results revealed that solvents known as prohibited non-standard LC solvents such as MtBE, DCM and THF in which the amylose derivatives CSP can be dissolved/swollen can be used as eluents in mobile phase compositions. The addition of these solvents will be also beneficial when used as diluents to directly monitor organic reactions online. Several tested racemates that were not separable under normal standard organic solvents were separated under non-standard organic solvents in mobile phase composition. The use of reversed phase consisting of ACN/H_2O broaden the application of CHIRALPAK IG® with enhanced resolution R_s and separation factor α comparing to similar separation under standard and non-standard organic solvents. Compared with CHIRALPAK IA® and in terms of resolution Rs and separation factor α, CHIRALPAK IG® appears to be superior under standard and non-standard solvents for the tested compounds. Overall, for the tested compounds, CHIRALPAK IG® appears to be superior to CHIRALPAK IA® and it may offer an alternative to CHIRALPAK IA®.

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